In Vivo Bone Formation by Human Bone Marrow Stromal Cells: Effect of Carrier Particle Size and Shape

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Received 8 February 2000; accepted 1 August 2000

Abstract: Successful closure of bone defects in patients remains an active area of basic and clinical research. A novel and promising approach is the transplantation of human bone marrow stromal cells (BMSCs), which have been shown to possess a significant osteogenic potential. The extent and quality of bone formation by transplanted human BMSCs strongly depends on the carrier matrix with which cells are transplanted; to date, hydroxyapatite/tricalcium phosphate (HA/TCP) supports far more osteogenesis than any other matrix tested. In order to further improve the technique of BMSC transplantation, we studied whether commercially available HA/TCP particles, clinically approved as an osteoconductive material and commercially available as particles measuring 0.5–1.0 mm diameter, is an optimum matrix for promoting bone development by BMSCs. HA/TCP and HA particles of varying size were sieved into a variety of size ranges, from <0.044 mm to 1.0–2.0 mm. Transplants were formed by mixing 40 mg aliquots of particles with cultured passaged human BMSCs. They were placed in subcutaneous pockets in immunocompromised Bg-Nu-XID mice and harvested 4 or 10 weeks later. The transplants were examined histologically; the presence of bone within each transplant was evaluated using histomorphometry or blindly scored on a semiquantitative scale. Transplant morphology and the amount of new bone varied in a consistent fashion based on particle size and shape. Transplants incorporating HA/TCP particles of 0.1–0.25 mm size demonstrated the greatest bone formation at both 4 and 10 weeks; larger or smaller particles were associated with less extensive bone formation, while a size of 0.044 mm represented a threshold below which no bone formation could be observed. Flat-sided HA particles measuring 0.1–0.25 mm formed no bone. The differences in bone formation were not attributable to the differences in cell attachment among the groups. Instead, the size and spatial and structural organization of the particles within BMSC transplants appear to determine the extent of bone formation. These findings provide necessary information for the successful clinical application of BMSC transplantation techniques. *Biotechnol Bioeng* **72:** 96–107, 2001.
Keywords: bone formation; bone marrow stromal cells; transplantation; hydroxyapatite-tricalcium phosphate

INTRODUCTION

Successful closure of bone defects remains a major concern to reconstructive surgeons. While most often secondary to trauma, bone loss can also arise from congenital disorders, neoplasms, and infections. A wide variety of materials have been employed to repair osseous defects, including autogenous cells, allogeneic tissues, and alloplastic materials. This variety of approaches attests to the absence of an optimal method for restoring bone integrity, especially in the presence of a sizable defect.

While surgeons have extensively used bone autograft to deliver osteoblasts and osteocytes to deficient sites, the transplantation of ex vivo expanded osteoprogenitor cells is a relatively recent and promising advance. Friedenstein (1973) and Owen et al. (1988) demonstrated the presence of a population of bone marrow-derived stromal cells with an osteogenic capability. These cells could be distinguished from the hematopoietic elements in the marrow by their high adherence to the substrate plastic in tissue culture flasks. They possess characteristics that are similar but not identical to connective tissue fibroblasts (Castro-Malaspina et al., 1980; Moreau et al., 1993; Penn et al., 1993). Cultured human bone marrow stromal cells (BMSCs) synthesize collagenous and noncollagenous proteins in vitro that are components of normal skeletal matrix (Benayahu et al., 1989; 1991). Following their expansion in tissue culture, the BMSCs from many species are capable of forming new bone when transplanted into immunocompromised recipient mice (Ashton et al., 1980, 1984; Friedenstein, 1973; Friedenstein et al., 1974; Gundle et al., 1995; Haynesworth et al., 1992; Krebsbach et al., 1997, 1999; Kuznetsov et al., 1989; Ohgushi et al., 1990, 1996; Thomson et al., 1993). In addition to heterotopic transplantation, BMSCs have been

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shown to repair induced bone defects in various animal models (Bruder et al., 1998a,b; Casabona et al., 1998; Krebsbach et al., 1998).

In order to make BMSC transplantation useful as a method for engineering new bone to close osseous defects in patients, the technique must be improved to optimize the growth rate, extent, and strength of newly formed bone. Attention has therefore been given to 1) methodologies which can modulate the differentiation potential and growth of these cells both ex vivo and in vivo, and 2) the development of a permissive matrix which can support the newly forming bone and perhaps even help induce bone formation. The requirements of a successful matrix are several. In addition to providing a nontoxic substrate on which cells can reside, it must be biocompatible with the host. It should possess a macromolecular structure which permits vascularization from the host tissue and allows the transplant to be incorporated into the surrounding tissue. The matrix should provide biomechanical qualities which match the biological role of the newly engineered tissue.

Optimization of the matrix is especially important in BMSC transplantation, since successful in vivo bone formation is heavily dependent on the relationship between the cells and matrix. Human BMSCs form bone when transplanted with hydroxyapatite/tricalcium phosphate (HA/TCP) particles or blocks, but fail to form comparable tissue when transplanted with porcine collagen sponges, polyvinyl sponges, poly(L-lactic acid), or demineralized bone matrix (Krebsbach et al., 1997). In contrast, mouse BMSCs form bone equally well in the presence of either HA/TCP or porcine collagen. Even the macroscopic qualities of the matrix are important. When comparing HA/TCP blocks with HA/TCP particles derived from the same blocks, human BMSCs form less extensive and poorer quality bone when incorporated into blocks than when incorporated into particles (Krebsbach et al., 1997). Whether this is due to differences in cell seeding of the HA/TCP or to differences in vascularization of the transplants has yet to be determined.

Just as formulation of the matrix in block or particulate form can heavily influence the final tissue product, changes in the macroscopic characteristics of the HA/TCP particles themselves may likewise influence the degree of bone formation. The two most important characteristics are particle size and shape. Until now, only HA/TCP particles measuring 0.5–1.0 mm in diameter have been transplanted in conjunction with BMSCs, because these particles are already approved for clinical use as an osteoconductive material (Krebsbach et al., 1997). In this study, we created a spectrum of particle sizes based on the original HA/TCP. These particles were roughly spherical in shape. Additionally, we created new HA particles which were flat-sided and whose sizes matched that of HA/TCP. These particles were transplanted with human BMSCs into recipient mice and the type and extent of bone which formed was evaluated. We also evaluated the extent to which BMSCs adhere to particles of different size, in order to explain differences in bone formation.

MATERIALS AND METHODS

Preparation of Carrier

Block HA/TCP (Zimmer, Warsaw, IN) was manually crushed to achieve particles of varying size. Using a sieve shaker (CSC Scientific, Fairfax, VA), particles were separated into size ranges including 1.0–2.0 mm, 0.5–1.0 mm, 0.25–0.5 mm, 0.1–0.25 mm, 0.062–0.1 mm, 0.044–0.062 mm, <0.1 mm, and <0.044 mm. Following sterilization at 200°C overnight, the particles from each size range were divided into 40-mg aliquots.

Additionally, a separate sample of HA particles was prepared by precipitation from aqueous solution at 22°C. This HA was formed by drop-wise addition of 0.4 L of a 0.0960 mol/L H_3PO_4 solution to a stirred 3.2 L 0.0200 mol/L solution of $Ca(OH)_2$ initially at pH 12.4 in a 3.8 L polypropylene bottle over 8 h followed by stirring and aging for three days after the reaction at pH 9.3. The $Ca(OH)_2$ solution was prepared by first heating 6.4028 g of $CaCO_3$ contained in a 10 ml Pt crucible at 1050°C for 4 h, cooling for 10 min in a desiccator, and then quantitatively dumping the CaO into water in the reaction bottle. The product (theoretical yield, 6.43 g) was collected by centrifugation without washing, lyophilized, and then analyzed by physical and chemical methods. TEM showed that the solid particles had irregular fibrous morphology with sizes about 50–200 nm in length and 7–30 nm in width. This HA was pressed into disks about 0.3 mm thick in a 25.4 mm diameter die under a total force of 124,544 N (28,000 pound force). The disks were ground in a mortar and sieved into a size range of 105–250 micron. The interparticle cohesion of these pressed and ground particles was tested by immersing them in water for 30 min at 22°C and by heating them at 150°C in air for 3 h; no physical changes in these particles were evident by either treatment under 50× optical examination.

Combined infrared, Raman, and X-ray diffraction analyses identified the HA product as poorly crystallized, containing carbonate and water impurities and essentially no HPO_4. The particles were found to have a Ca/P ratio of 1.66, a carbonate content of 0.7 mass %, a water content of about 5 mass %, a HPO_4 content of <1% of the total PO_4, and a surface area of 113 m^2/g by the BET method (Brunauer et al., 1938). Similarly, the Zimmer HA/TCP particles have a Ca/P ratio of 1.6, a carbonate content of 0.9%, a water content of <0.5 mass %, an estimated HPO_4 content of <0.5% of the total PO_4. Of note, the surface area of the Zimmer particles was approximately 1–6% of that of the HA particles, based on a Zimmer base particle size varying from 0.3–2.0 micron. SEM and optical analyses revealed that the HA ground particles were plate-like in morphology, the surfaces were smooth, and pores, if present, were less than about 0.2 micron in diameter. In contrast, HA/TCP particles from Zimmer were irregularly shaped and rough in appearance; 1 mm diameter particles had surface pores measuring 200–300 micron in diameter and 400–600 micron in depth.

Cell Culture and Transplant Preparation

Surgical specimens containing fragments of normal unaffected bone with bone marrow were obtained from a single 7-year-old otherwise healthy female patient undergoing reconstructive surgery. Tissue procurement, under NIH IRB-approved protocol 94-D-0188, proceeded in accordance with NIH regulations governing the use of human subjects. Multicolony-derived strains of BMSCs were obtained from the bone marrow in a manner previously described (Kuznetsov et al., 1997). Briefly, bone marrow cells were cultured in growth medium consisting of αMEM, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 10^{-8} M dexamethasone, 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate, and 20% fetal bovine serum of a preselected lot. Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2.

Trypsin-released cells from passages 3 through 5 were pipetted into 1.8 mL polypropylene cryotubes, each previously loaded with a 40 mg aliquot of HA/TCP particles. Each tube received either 3.6×10^6 or 5.0×10^6 cells. The mixtures were incubated for 90 min at 37°C on a slowly rotating platform. An aliquot of media from each tube was sampled to determine the number of cells which had not attached to particles. The mixtures were then centrifuged at 200*g* for 60 sec and the supernatant again sampled. The remaining supernatant was discarded. Fifteen μL aliquots of mouse fibrinogen (Sigma, St. Louis, MO), which had been reconstituted in water, and 15 μL of mouse thrombin (Sigma) reconstituted in 2% $CaCl_2$, were added to each transplant mixture and allowed to form a gelatinous solid. Because the extent and character of new bone formation was the focus of this study, no cell-free or particle-free transplants were created. Subcutaneous HA/TCP transplants alone, as well as transplants of cultured BMSCs without a carrier matrix, fail to form bone (Krebsbach et al., 1997, 1999).

Three-month-old immunodeficient Bg-Nu-XID female mice (Harlan-Sprague Dawley, Indianapolis, IN) served as transplant recipients. Twenty mice were given a total of 72 transplants. Each mouse had multiple transplants, but at most one of each particle size tested. A balance among subset of particle size transplants was maintained within each group of mice. It was logistically impossible to administer transplants for all nine types of particle sizes within the same animal. All but one mouse survived both the operation and intended postoperative follow-up period.

Operations were performed in accordance to specifications of an approved NIH small animal protocol (97-024). All animals were cared for according to the policies and principles established by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Mice were anesthetized with a combination of IP ketamine (140 mg/kg body weight) and IP Xylazine (7 mg/kg body weight). Transplants were placed in the subcutaneous tissues beneath the dorsal skin through a midline longitudinal skin incision. Incisions were closed with surgical staples. The mice were sacrificed at 4 or 10 weeks postoperatively and their transplants harvested.

The transplants were fixed in either 4% phosphate-buffered formalin freshly prepared from paraformaldehyde (PBF) or Bouin's. PBF-fixed transplants were demineralized in buffered 10% EDTA. Each transplant was sectioned into two pieces through its midline and embedded in paraffin so that its largest surface areas were sectioned. Six sections were obtained from each transplant through its center. Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin.

Estimates of Bone Formation

Bone formation within the transplant was estimated using two methods. First, bone formation was blindly, semiquantitatively estimated by three independent evaluators in a manner similar to that described previously (Kuznetsov et al., 1997). Transplants were scored on a scale of 0 to 4; a score of 0 corresponded to no bone formation, while a score of 4 was given to transplants with abundant bone formation occupying greater than one-half of the transplant (Table 1). The average of the three separate bone formation scores was calculated for each transplant and particle size. The average bone scores were analyzed using a one-way analysis of variance procedure in which particle size was included as a fixed effect. Comparisons were made for pairs of transplant groups having distinct particle sizes, using the pooled estimate of variation in bone scores. Comparisons were also made between bone scores for transplants of different particle sizes using the paired *t*-test among subgroups of animals that received both particle size transplants, whenever possible. Statistical analyses employed the SAS software package, ver. 6.12 (SAS Institute, Cary, NC).

Second, bone formation was quantitatively estimated using a histomorphometric image analysis system (Bioquant; R&M Biometrics, Nashville, TN) and a Zeiss microscope at ×12.5 and ×25 magnification. Within each section, measurements were made of the entire transplant (T), the bone area (B), and the particle area (P). Two measures, the fraction B/T of bone area (B) in relation to total transplant area (T), and the fraction B/(T-P) of bone area (B) in relation to the area of the transplant (T) minus the particles (P), served as the response measures. We derived the values B/(T-P) to

Table I. Semiquantitative scale for the estimation of bone formation.

Score	Extent of bone present within the transplant
0	No bone evident
1	Minimal bone evident (1 trabecula)
2	Weak bone formation, occupying only a small portion of the transplant
3	Moderate bone formation, occupying a significant portion but less than one half of the transplant
4	Abundant bone formation, occupying greater than one half of the transplant

determine whether exclusion of the particle area from the histomorphometric calculations would associate a different set of particles with the greatest bone formation. The square root transformation was used for these histologically based bone measurements to stabilize the variances among groups having different particle size transplants. These responses were analyzed using a one-way analysis of variance model in which particle size was included as a fixed effect. Comparisons were made for pairs of transplant groups having distinct particle sizes, using the pooled estimate of variation in histologically based bone scores. Comparisons were also made between the histologically based bone scores for transplants of different particle sizes using the paired *t*-test among subgroups of animals that received both particle size transplants.

Correlations were evaluated between the semiquantitative and histologically based measures of bone formation. Regression models were fitted to predict the histologically based levels of bone formation based on the semiquantitative clinical bone scores. Again, the square root of the histologically based bone scores was modeled directly.

Cell Adhesion Analysis

We determined whether the number of cells attaching to the particles were responsible for the differences in bone formation between transplants of different particle size. Aliquots of media with nonattached cells were collected from cryotubes containing cells and particles which had been incubated for 90 min, before and after centrifugation. A 50-μL sample of media was combined with 10 mL of counting solution containing Isoton and 1% FBS. Cell counts were performed using a Coulter Counter (Coulter Electronics, Hialeah, FL). A trypan blue viability assay was performed on a subset of samples; cell viability averaged 97%. The percentages of cells adhering to HA/TCP particles were analyzed using a one-way analysis of variance model in which particle size was included as a fixed effect. All statistical analyses were performed with the SAS® software.

RESULTS

The average number of transplants per mouse was four, representing 72 total transplants. Each particle size transplant was tested in three to seven animals. Six mice were harvested at 4 weeks and 13 mice at 10 weeks postoperatively. All but one mouse survived the experiment. Since all particle size transplants were not present in any one animal, direct comparisons between distinct particle size transplants, adjusted for individual animal effects, were performed within subsets of animals. Unadjusted comparisons among particle size transplants were made for combinations of different animals.

Transplant Morphology

Bone morphology and the spatial arrangement of the particles varied with particle size and type. Transplants employing the largest HA/TCP particles (1.0–2.0 mm) were characterized by particles widely separated by nonmineralized tissue (Fig. 1A). Bone was sporadic and discrete and associated with individual particles. Where it occurred, the bone was lamellar in character. Hematopoietic tissue was sparse. This was true as well for transplants with the second-largest HA/TCP particles, 0.5–1.0 mm (Fig. 1B). In contrast, transplants with HA/TCP particles measuring 0.25–0.5 mm and 0.1–0.25 mm contained abundant bone and associated hematopoietic tissue and occasional adipocytes (Fig. 2A,B). Particles were closely spaced, with bone occupying much of the interparticle space. In many areas, bone associated with individual particles appeared to coalesce, forming a rim of bone along the exterior surface of the transplant and a latticework of bone within the transplant. Transplants with slightly smaller HA/TCP particles, measuring 0.062–0.1 mm, demonstrated well-developed rim and lattice-like bone which was limited to a few discrete areas, while the remainder of the transplants were occupied by fibrous tissue (Fig. 2C). Transplants with the smallest HA/TCP particles, those measuring either <0.1 mm, 0.044–0.062 mm, and <0.044 mm formed no bone (Fig. 3A–C). Of greatest interest, transplants utilizing HA particles of size 0.1–0.25 mm formed no bone and exhibited limited vascularization. In contrast to similarly sized particles of HA/TCP, the 0.1–0.25 mm HA particles were flat-sided; they formed arrangements resembling closely packed stacks of plates (Fig. 3D). All transplants and peritransplant tissues were characterized by the absence of an inflammatory reaction.

Timing and Extent of Bone Formation

Significant amounts of new bone was observed as early as 4 weeks in transplants with particles as large as 2.0 mm and as small as 0.062 mm ($P < 0.001$, Fig. 4). The extent of bone formation observed as a function of transplant particle size was fairly stable, but with some variability for the 4-week evaluation ($P > 0.05$ for all comparisons) above an apparent particle size threshold of roughly 0.05 mm (Fig. 4).

Bone formation was increased significantly in all groups except the 0.044–0.062 group at 10 weeks (Fig. 4). The extent of bone formation varied as a quadratic function of particle size after 10 weeks, using either semiquantitative bone scores (Fig. 4) or histomorphometric evaluation (Fig. 5). Transplants incorporating HA/TCP particles of 0.1–0.25 mm size demonstrated the greatest bone formation, closely followed by transplants incorporating particles ranging in size from 0.25–0.5 mm. Further increasing particle size resulted in diminishing bone formation, although the largest particle size was still associated with moderate amounts of bone. Particles ranging in size from 0.044–0.1 mm were associated with weak to moderate bone formation, while particles smaller than 0.044 were associated with no bone formation. Among HA/TCP particles, a size of 0.044 mm represented a threshold below which no bone formation could be observed. Significant differences in the extent of

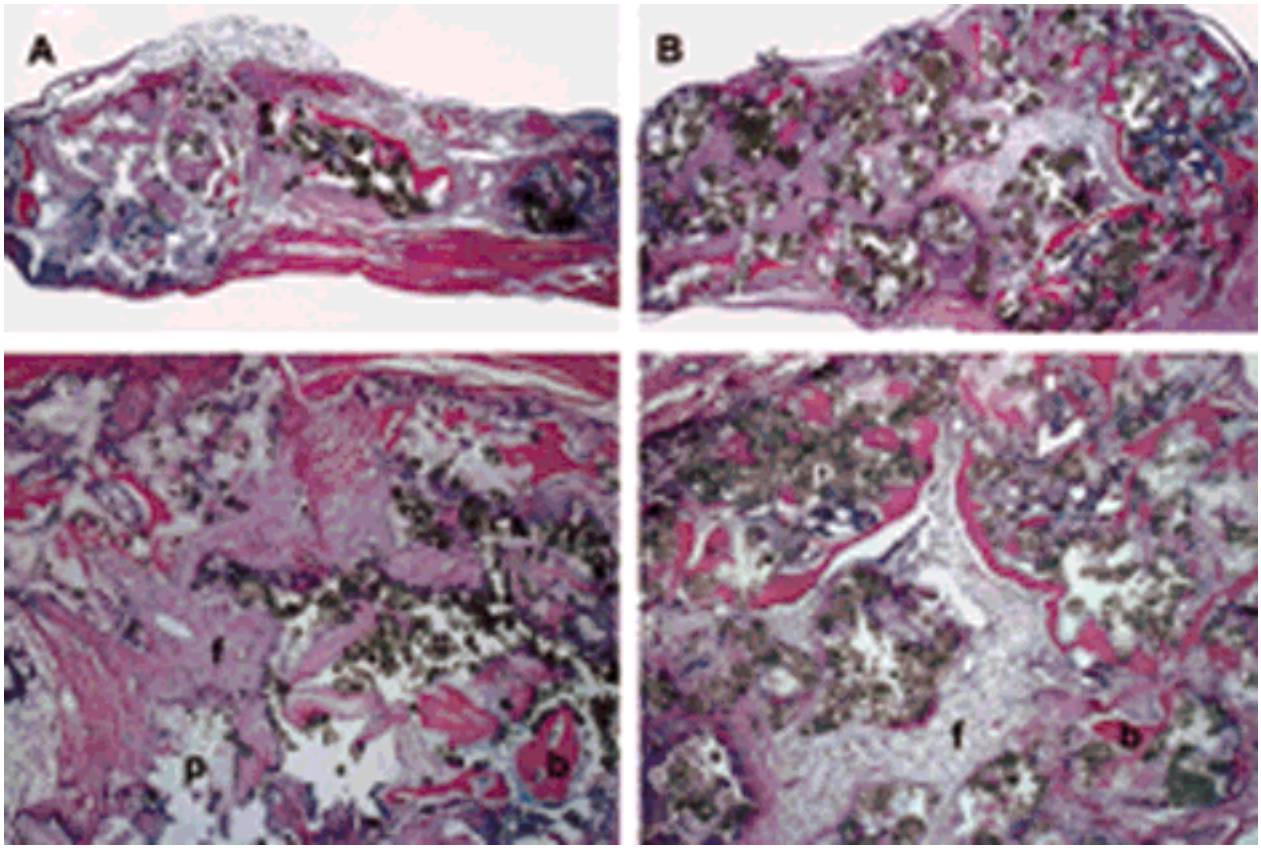

Figure 1. Transplants with the two largest sized particle groups. **A:** Transplant with particles measuring 1.0–2.0 mm. Particles around which bone has formed are widely separated by connective tissue. **B:** Transplant with particles measuring 0.5–1.0 mm. Particles are closer together, although new bone does not bridge adjacent particles. (b = bone, f = fibrous connective tissue, p = particle.) Magnification: upper image 25×, lower image 50×. Stain: Hematoxylin and eosin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bone formation were observed between the 0.1–0.25 mm and the 0.5–1.0 mm or 1.0–2.0 mm particle size transplants using either the clinical bone scores or histologically based measures. No significant difference could be detected between the 0.1–0.25 mm and the 0.25–0.5 mm particle size transplant groups. Transplants incorporating HA particles, of which only the 0.1–0.25 mm size were studied, were associated with no bone formation.

In all transplants that had undergone histomorphometric analysis of bone formation, there were statistically significant differences among the mean scores in the pooled particle size groups for the transformed B/T and B/(T-P) measures (Fig. 5). Again, a quadratic response gradient was observed for each measure as a function of particle size up to a size of 0.1–0.25 mm; this tapered off rapidly to that observed for particles of size 1.0–2.0 mm. This pattern held separately for each of the two measures investigated. The maximum bone formation was observed for the 0.1–0.25 mm sized particles for each response measure. Each bone formation measure (B/T and B/[T-P]) for this particle size was significantly larger than those for either the 0.5–1.0 mm or the 1.0–2.0 mm particle size groups ($P < 0.05$). The bone measures for the 1.0–2.0 mm group were smaller than those for the 0.5–1.0 mm group ($P = 0.0507$). For the paired *t*-tests in which particle size group comparisons were made adjusting for individual animal effects, significant differences were detected between the 1.0–2.0 mm and the 0.5–1.0 mm group, 0.25–0.5 mm group, and the 0.1–0.25 mm group, separately. The findings from the paired *t*-tests generally paralleled those found for the unadjusted comparisons.

Comparison of Histomorphometric Data with Semiquantitative Bone Measurements

The transplant bone scores were compared to the histomorphometric measurements to determine whether the semiquantitative bone score estimates represent a quantifiable amount of bone. A relationship between bone scores and histomorphometric data, including B/T and B/(T-P), is depicted in Figure 6. The correlations between the semiquantitative bone scores and the B/T and B/(T-P) histological measures were 0.876 and 0.864, respectively. When the square root of the fraction of bone area in relation to the area of the transplant (B/T) and the semiquantitative bone scores were compared, a stronger association was observed (r = 0.973). The estimated functional relationship between these measurements is described as:

$$100 * \text{B/T} = [0.944 * \text{bone score} - 0.088]^2$$

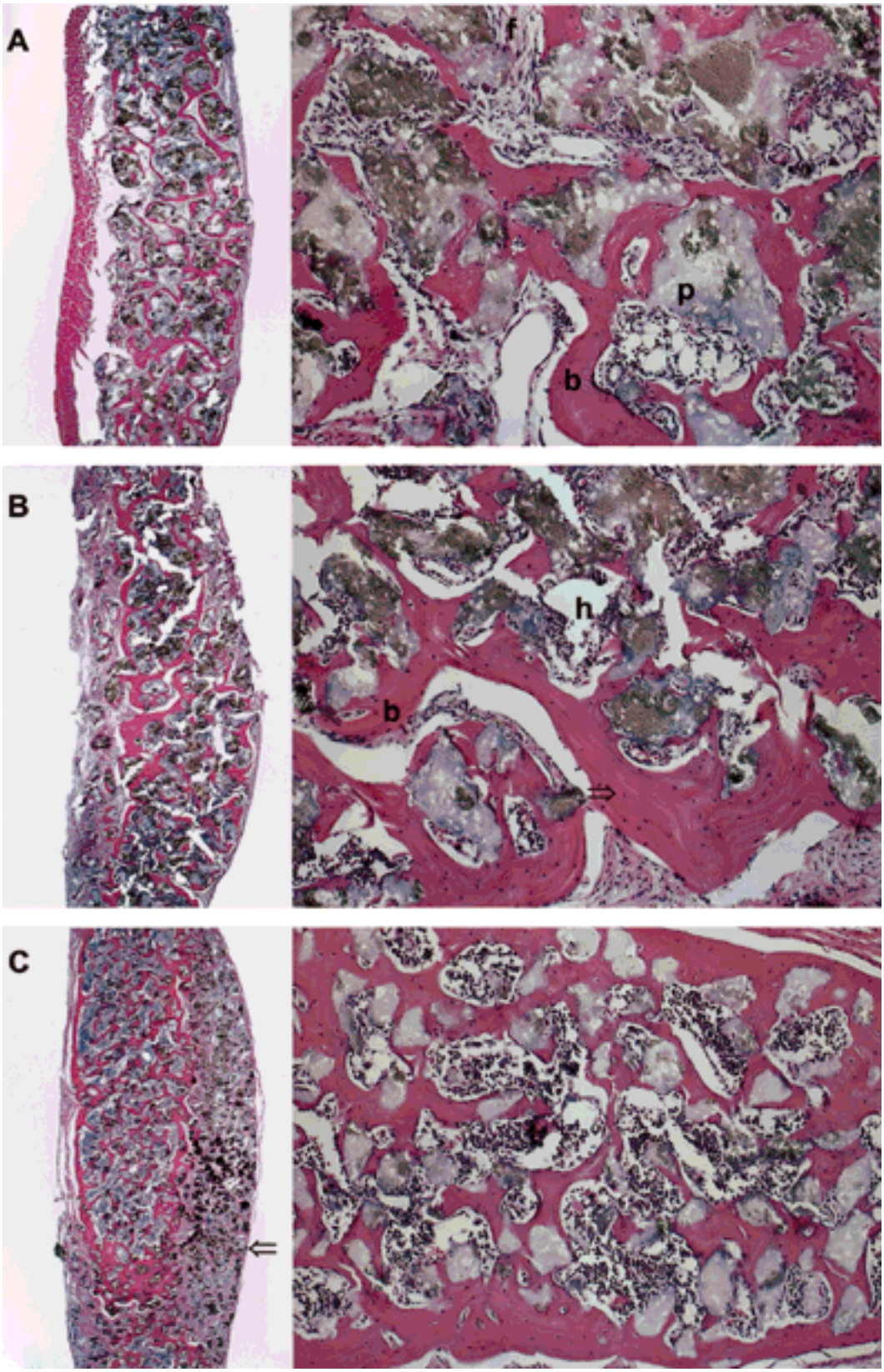

Figure 2. Transplants which formed extensive bone. **A:** Transplant with particles measuring 0.25–0.5 mm. In contrast to the largest particles, this transplant has extensive bone and only minimal fibrous connective tissue. Hematopoiesis is present but not extensive. **B:** Transplant with particles measuring 0.1–0.25 mm, which have the most extensive bone. Lamellar bone is easily visualized here (⇒), although it can also be seen in **A** and **C. C:** Transplant with particles measuring 0.062–0.1 mm. Portions of the transplant are devoid of new bone (⇐). New bone closely joins multiple particles in a dense latticework and forms a cortical shell. (b = bone, f = fibrous connective tissue, p = particle, h = hematopoietic tissue.) Magnification: left image 25×, right image 100×. Stain: Hematoxylin and eosin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

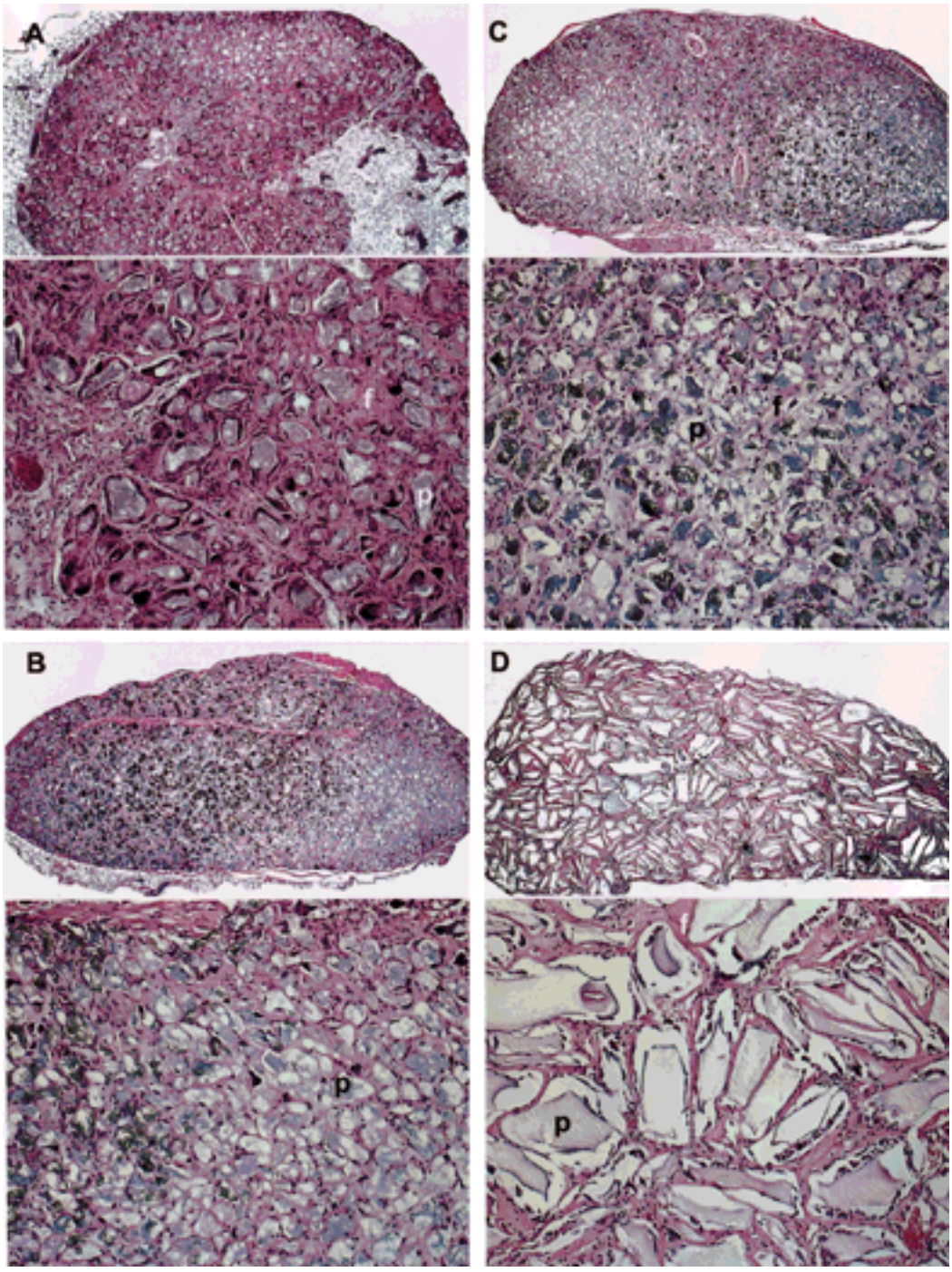

Figure 3. Transplants which formed no bone or hematopoietic tissue. **A:** Transplant with particles measuring <0.1 mm. **B:** Transplant with particles measuring 0.044–0.062 mm. Note the minimal tissue between particles. **C:** Transplant with particles measuring <0.044 mm. **D:** Transplant utilizing HA particles of size 0.1–0.25 mm. Particles are closely stacked, with only a thin film of tissue between particles. (f = fibrous connective tissue, p = particle.) Magnification: upper image 25×, lower image 100×. Stain: Hematoxylin and eosin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

A similarly strong association was also observed between the square root of the fraction of bone area in relation to the area of the transplant, excluding the area of particles (B/[T-P]) and the bone scores ($r = 0.970$). This estimated functional relationship is described as:

$$100 * B/(T\text{-}P) = [1.171 * \text{bone score} - 0.123]^2$$

Using these relationships, we estimated the histomorphometry values [$100 * B/T$ and $100 * B/(T\text{-}P)$] for each of the five bone scores together with their associated 95% confidence intervals (Table 2).

Although we believed that theoretically a bone score of zero should be associated with histomorphometric values of zero, for statistical reasons we fit a model which allowed for

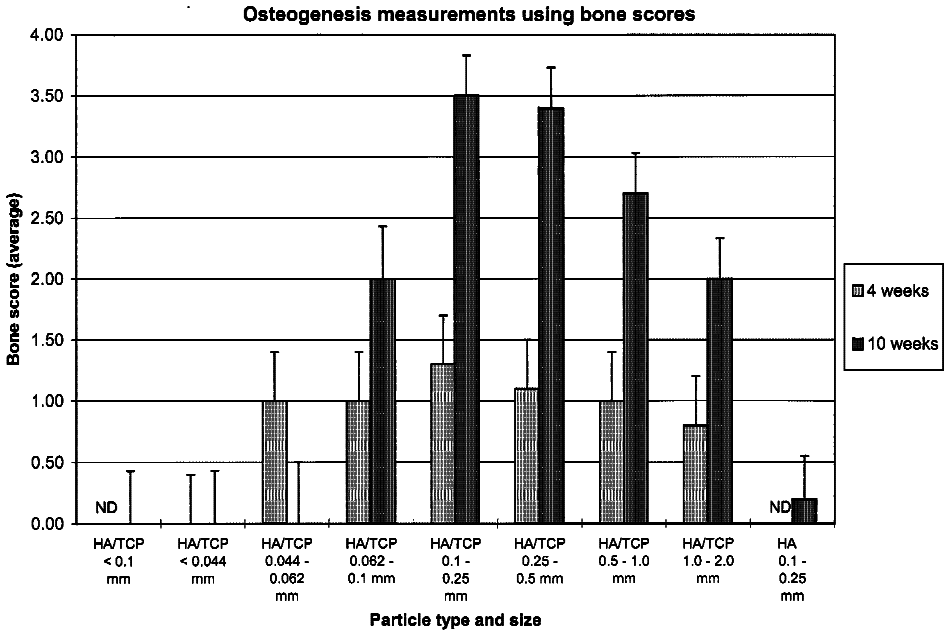

Figure 4. Osteogenesis by BMSCs in transplants with HA/TCP particles of varying size, evaluated at 4 and 10 weeks following transplantation. Bone scores from three independent observers of transplants from all mice were pooled. Each particle size's mean bone scores were compared to those of the HA/TCP 0.1–0.25 mm particles to investigate significant differences. Bars represent standard error of the mean. Nd = Not done. * = statistical significance at $P < 0.05$ level. # = statistical significance at $P < 0.001$ level.

a nonzero intercept. The estimates of the intercept that we derived are trivially different from 0, since $0.088^2 = 0\%$, and $0.123^2 = 0.02\%$, a further validation of these relationships.

Cell Adhesion Analysis

In order to determine whether differences in bone formation are caused by differences in cell attachment, unattached cells were counted in the supernatant of transplants which incorporated three sizes of particles: 0.5–1.0 mm, 0.1–0.25 mm, and 0.044–0.062 mm. The largest particles have the smallest surface area per mg of particle, while the smallest particles have the largest surface area. Assuming each particle is a perfect sphere, an aliquot of particles measuring 0.044–0.062 mm has approximately 15 times the surface

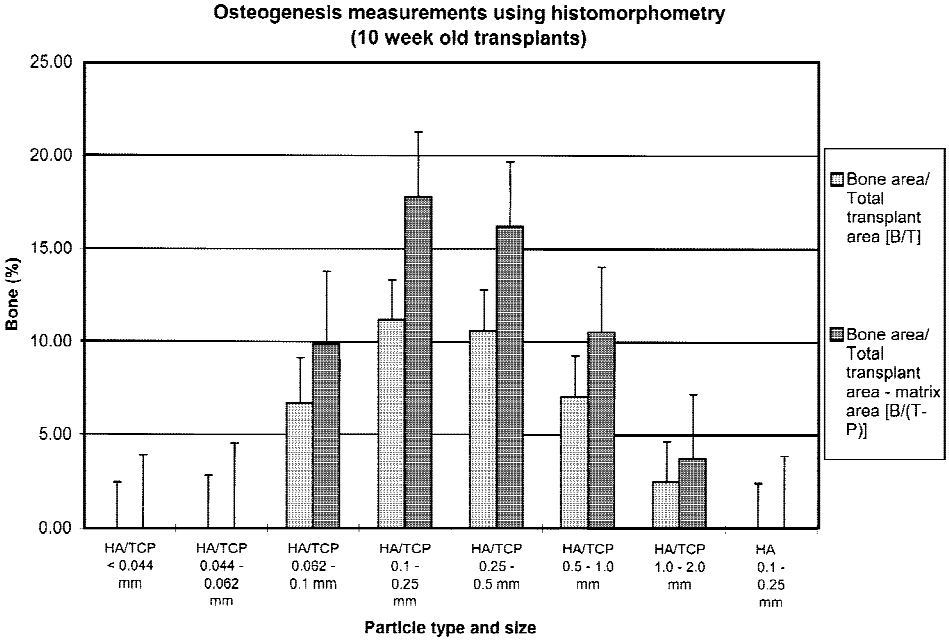

Figure 5. Osteogenesis by BMSCs in transplants with HA/TCP particles of varying size, evaluated 10 weeks following transplantation. Both B/T and B/(T-P) values were calculated. Histomorphometry data from all mice were pooled at each particle size. Each particle size's mean histomorphometry values were compared to those of the HA/TCP 0.1–0.25 mm particles to investigate significant differences. Bars represent standard error of the mean. * = statistical significance at $P < 0.05$ level. $ = statistical significance at $P < 0.01$ level.

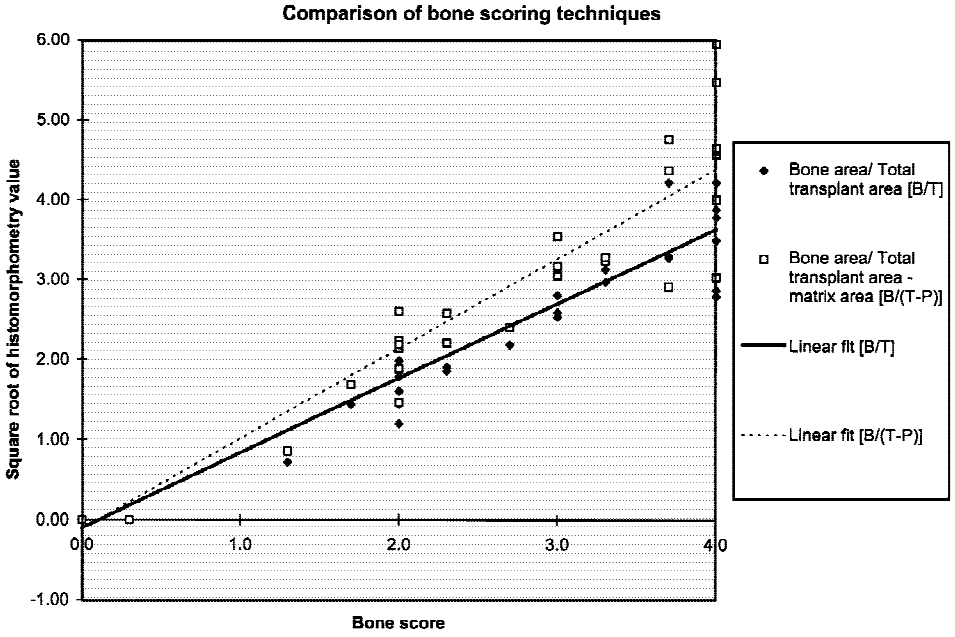

Figure 6. Comparison of the square root of the histomorphometry values with bone scores from the same transplants.

area of an identically massed aliquot of particles measuring 0.5–1.0 mm and approximately 4 times the surface area of an identically massed aliquot of particles measuring 0.1–0.25 mm.

Cells were counted immediately following the 90-min attachment phase, both before and after centrifugation. Among these three groups of transplants, those with the largest sized particles (0.5–1.0 mm) had the fewest percentage of attached cells, while those with the smallest particles (0.044–0.062 mm) had the greatest percentage of attached cells (Fig. 7). By keeping the mass of the transplants constant, an aliquot of 0.5–1.0 mm HA/TCP particles had fewer particles and less surface area than an aliquot of 0.044–0.062 mm particles. Decreasing particle size was therefore associated with increasing levels of cell attachment, both before and after centrifugation.

DISCUSSION

Previously, Kuznetsov et al. (1989) transplanted BMSCs under the renal capsule in mice and noted bone formation within the transplants. They characterized the bone as lamellar, with long trabeculae and abundant hematopoiesis. While their system provided the first method for transplanting BMSCs outside a diffusion chamber, its major shortcoming was its limitation to kidney transplantation. Goshima et al. (1991a,b,c) expanded transplantation beyond the renal capsule by successfully obtaining bone formation within the subcutaneous space. They seeded rat or quail BMSCs on porous HA/TCP blocks, placed the blocks un-

Table II. Predicted histomorphometric values for each bone score, together with their associated 95% confidence intervals.

Bone score	Bone area/total transplant area [B/T] (%)	Bone area/total transplant area—matrix area [*B/(T-P)] (%)
0	0.00 [0, 0.07]	0.0 [0, 0.01]
1	0.73 [0.52, 0.98]	1.10 [0.77, 1.49]
2	3.24 [2.83, 3.68]	4.92 [4.29, 5.55]
3	7.53 [7.02, 8.05]	11.49 [10.72, 12.28]
4	13.60 [13.14, 14.07]	20.8 [20.16, 21.46]

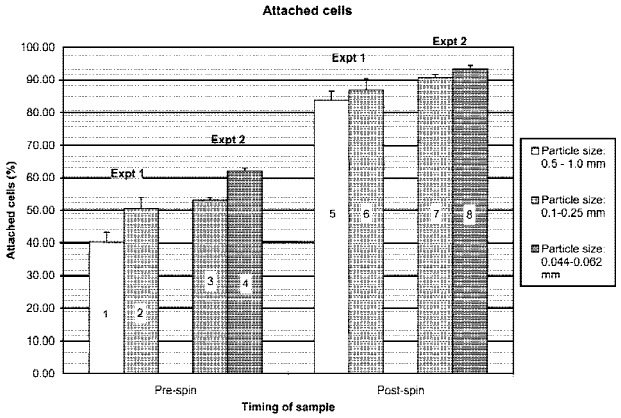

Figure 7. Percentage of cells attached to HA/TCP particles following a 90-min incubation. This portion of the study included two separate experiments; each experiment compared one set of particles to a set of particles measuring 0.1–0.25 mm. Experiment 1 compared particles measuring 0.5–1.0 mm with those measuring 0.1–0.25 mm, while Experiment 2 compared particles measuring 0.1–0.25 mm with those measuring 0.044–0.062 mm. In both experiments, cell attachment increased as particle size decreased. Samples were evaluated both before (prespin) and after (postspin) centrifugation. The difference between columns 1 and 2 and between columns 5 and 6 is statistically significant at a $P < 0.05$ level. Bars represent standard error of the mean.

derneath the skin of rat recipients, and observed bone formation in the pores of the blocks by as early as 2 weeks. Krebsbach et al. (1997) extended Friedenstein's work by transplanting human BMSCs into the subcutaneous space of mice. They evaluated a variety of carriers, determining which facilitated the greatest bone formation by BMSCs, and they developed a method for transplanting these constructs under the mouse skin. Using in situ hybridization and immunohistochemistry, they confirmed that 8-week-old transplants derived their bone from donor cells while their hematopoietic elements came from the recipient animal.

BMSCs must be transplanted in association with a carrier, or matrix, in order to form bone in vivo. While certain carriers have been identified as appropriate for bone formation by human BMSCs, others have been found to be appropriate for mouse BMSCs but not for human cells. Mouse BMSCs transplanted in collagen and polyvinyl sponge matrices formed a capsule of cortical-like bone which surrounded a core of active hematopoiesis. Consistent bone formation by human BMSCs was achieved only within carriers containing HA/TCP ceramics in the form of blocks, powder, and powder mixed with Type I bovine fibrillar collagen strips (Collagraft™). Notably, bone formation was insignificant among transplants that included Gelfoam™ or polyvinyl sponges infiltrated with human BMSCs.

These studies utilized a commercially available HA/TCP marketed as an osteoconductive material for clinical application. When mixed with autologous bone marrow and bovine collagen, this HA/TCP swerves as a bone graft extender. It has been successfully used to treat acute long bone fractures (Kocialkowski et al., 1990) and delayed unions and nonunions of long bone fractures (Cornell et al., 1991). These HA/TCP particles range in size from 0.5–1.0 mm diameter. While this size range and shape is effective in the role for which it has been prescribed, it may not be optimum as a vehicle for promoting bone development by BMSCs.

In this study, we moved a step closer towards clinical utilization of BMSCs by examining the contribution of HA/TCP particle size and shape to bone formation. We created a spectrum of particle sizes based on the original, spherical HA/TCP. All HA/TCP particles, regardless of diameter, were derived from an original, crushed sample of porous block HA/TCP; they therefore exhibited a consistent surface texture. Our data demonstrate that HA/TCP particle size plays a crucial role in determining the extent of bone formation by transplanted human BMSCs. We observed a quadratic, unimodal relationship between the size of spherical particles and bone formation, with peak bone formation at 0.1–0.25 mm. Below a threshold of 0.044 mm, no bone formed; the smallest particles may have impeded transplant vascularization due to their close packing. Bone was observed among transplants incorporating the largest particle size studied, 1.0–2.0 mm, but the amounts were substantially less than those observed for the 0.1–0.25 mm particle size group. These observations were present at both time points (4 and 10 weeks posttransplantation). No transplants exhibited evidence of particle-mediated toxicities. Additionally, we created new flat-sided HA particles whose sizes matched those of the spherical HA/TCP. Transplants with flat-sided HA particles measuring 0.1–0.25 mm diameter, in which the particles rested closely together, failed to produce bone and exhibited limited transplant vascularization. This was most likely secondary to the low porosity of the particles or to their close packing. We hypothesize that close particle packing, a by-product of particle shape, may modulate bone formation by impairing transplant vascularization.

Interestingly, the most extensive and complex latticework of new bone formed in those transplants with particle sizes measuring 0.1–0.25 mm and 0.062–0.1 mm. The bone fixed adjacent particles to each other. We would expect this to increase the tensile strength of the transplants, although we have not performed biomechanical tests on these specimens. This ability to form a rigid form with continued bone development highlights the advantage of a particle-based transplant system over transplants which utilize HA/TCP blocks. Block-based transplants form sparse bone at their periphery and even less bone in their interior, while particle-based transplants have abundant bone formation throughout their cross-section (Krebsbach et al., 1997).

Some of the differences that we observed in average bone formation scores between distinct particle size groups whose transplants formed significant bone were not statistically significant. We feel that this absence of a statistically significant difference is specifically attributable to the small power inherent in this study due to the small number of transplants (seven or less) per particle size at each time point. In order to confirm this point, we reanalyzed our data with one modification—we doubled the sample sizes by repeating each observation twice. The effect measures remain identical, but the precision is increased. This two-fold modification resulted in a statistically significant difference between the particle size 0.1–0.25 mm and the sizes 1.0–2.0 mm, 0.5–1.0 mm, 0.062–0.1 mm, and 0.044–0.062 mm. Since our original study was more exploratory by design, specific power calculations to determine the number of animals per group to detect such differences in bone formation were not performed. However, the observed numerical differences were substantial, and some statistically significant differences were observable among different particle size transplants, even with very small numbers of animals per group. Confirmation of our findings should be possible by expanding the study groups, coupled with more efficient allocation of transplants per animal. Based on these findings, one should be able to show significant differences among the major particle size transplants by increasing the number of animals by 50%, provided one incorporates a reasonably connected design.

While no previous publications evaluate the effect of particle size on in vivo BMSC differentiation, a few studies describe the in vitro effect of HA/TCP particle size on fibroblast cell proliferation (Cheung et al., 1997; Sun et al., 1997), collagenase activity (Cheung et al., 1997), fibroblast and myoblast secretion of TGFβ-1 and PGE_2 (Sun et al., 1997), and osteoblast proliferation and secretion of TGFβ-1

and PGE_2. These studies suggest that particles measuring <0.1 mm are poorly tolerated by cells. Transplantation of HA/TCP particles alone into hard tissue defects in rabbits and dogs was associated with superior bone formation around particles measuring 0.3–0.6 mm, while larger and smaller particles were associated with less bone formation (Higashi et al., 1996; Kuroda, 1995). These results are in agreement with our findings.

Relationship of Cell Attachment to Particle Size and Bone Formation

In an attempt to describe the mechanism by which particle size influences bone formation, we studied the attachment of BMSCs to particles of different sizes. The number of cells attaching to particles is not directly responsible for the differences in bone formation between particles of different sizes. In these studies, an increase in particle size was associated with a decrease in cell attachment. Aliquots of the largest particles, 0.5–1.0 mm diameter, captured only 40% of cells during the incubation period, while the particles measuring 0.044–0.062 mm captured 62% of cells. Yet the larger particles were associated with a reasonable degree of bone formation, while the smaller particles were associated with essentially no bone formation. The greater number of cells captured by the smaller particles apparently offered no advantage for bone formation. The larger particles captured fewer cells than the smaller particles because they offered less surface area per aliquot. Thus, it is not the number of cells attached to the particles that determines the fate of the transplants. Rather, osteogenic differentiation of transplanted BMSCs is influenced by the relative arrangement of ceramic and cells within the transplants; some empty space is necessary to allow capillary ingrowth, while excessive empty space favors development of nonmineralized fibrous tissue. A more precise mechanism by which particle size influences bone formation is yet to be described.

Histomorphometry vs. Semiquantitative Bone Measurements

Finally, the previous use of semiquantitative bone measurements was here validated by their comparison to histomorphometric results. As bone score increased, the average area of the transplant occupied by new bone increased accordingly. Bone scores of 0 represented no bone formation by histomorphometry, while a bone score of 4 indicated that bone occupied 13.6% and 21.2% of the cross-sectional area of the total transplant and the nonmatrix area of the transplant, respectively. It is our conclusion that semiquantitative bone scores are representative of total bone formation. Histomorphometric analysis, while providing more complete information, is much more labor-intensive; in cases where dozens of transplants are to be analyzed, semiquantitative scoring may be acceptable. Comparisons between histologic bone scores and histomorphometric measures of bone-containing ceramic cubes have also demonstrated the utility of subjective measures (Dennis et al., 1998). However, our bone scoring system differs from Dennis' in several ways. His method examines the percentage of bone-containing pores, whereas ours measures the absolute amount of bone present within the transplant. Additionally, our system describes an exponential relationship with a high r^2.

This study demonstrates that HA/TCP particle size and shape play a critical role in bone formation by transplanted human BMSCs, and it describes parameters which can be optimized to improve the clinical utility of the technique.

The authors thank Zimmer (Warsaw, IN) for its gift of HA/TCP.

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